CHARACTERIZATION OF PROTEASE INHIBITOR FROM THE SEEDS OF UNDER-UTILIZED LEGUME *Mucuna Pruriens*

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Introduction

The proteolytic activity of the proteases in the biological systems are regulated by various mechanisms. The activity of the proteases can be inhibited due to the self proteolytic degradation or binding of the protease inhibitors. Protease inhibitors are the proteins or peptides which inhibits the catalytic activity specifically or non-specifically of the proteases by forming highly stable stoichiometric complexes. This complex can be formed due to the binding of the protease inhibitor with the active site of the proteases by mimicking the structure of tetrahedral intermediates as homologous structure that occurs in an enzyme catalyzing reactions [1]. The existence of the protease inhibitors in nature was first reported by Fermi and Pernossi [2]. They are synthesized either as preproproteins or pre-proteins and during synthesis are processed to form native PIs [3–5]. The number and types of the proteases inhibitors reported so far are extremely large and hence forms a good system to study their various aspects like molecular evolution, structural and functional relationships etc.

The protease inhibitors controls the proteolytic events in all living organisms and are widely spread in nature. They are widely distributed in plants, animals and microorganisms [6]. Most of the protease inhibitors have been extensively purified and characterized from storage tissues such as seeds and tubers of plants belongs to garminaceae, fabaceae, solanaceae and poaceae families. They have also been reported from non-storage tissues like leaves, flowers and roots [7,8].

Plants being a potent source of protease inhibitors, a large number of protease inhibitors have been identified and characterized. They played an important role in mobilization of the storage proteins and regulates endogenous and exogenous proteolytic activities. These protease inhibitors will block the proteolytic activity of the proteases which causes a reduction of amino acids for their growth and development and hence acts as anti-nutritional factors [9]. In the aerial parts of the plant, these protease inhibitor molecules are responsible for several stimuli which have been widely reported. These molecules also participates in cell metabolism and also in many physiological processes of the plant [10,11].

Several types of serine protease inhibitors have been extensively purified, characterized, and assessed for their biological potentials from various sources. In plants, these proteins are involved in many physiological processes including regulation of endogenous and exogenous unwanted proteolysis activities, delaying senescence and acts as defensive molecules protecting the plant against pathogens [12,13]. They were also involved in signal transduction, mobilization of storage proteins, morphogenesis, and development of other organs [14].

More recently, serine protease inhibitors (SPIs) have received greatest attention from scientific community of biomedicine and biotechnology. They acts as an efficient tool to control unwanted proteolysis and also to treat diseases like AIDS, inflammation, signal cascade in immune system, cell cycle, hypertension, blood coagulation, respiratory diseases, antimicrobial, Cancer, and neuro-degeneration disorders like Alzheimer's diseases. Therefore, these could be a potent candidates for the drug design and development [10,15–17].

Serine protease inhibitors may play an effective role in treatment of inflammatory diseases by modifying various inflammatory pathways. Estrogen exposure accelerates pro-inflammatory responses in activated splenocytes. It has been shown that 4-(2-aminoethyl) - benzenesulfonyl fluoride (AEBSF), a serine protease inhibitor, suppresses inflammatory cytokines and related transcription factors in splenocytes from estrogen-treated mice [18].

The significance of regulated membrane-anchored and secreted serine proteases to maintain homeostasis and its relation with these enzymes and cancer reflects that these enzymes must be strictly controlled in normal physiological conditions. Therefore, enzymatic breakdown of serine proteases is considered to be one of the important regulators for maintaining cellular homeostasis. However, excessive enzymatic activity is often an adverse effect on the cellular processes, and this can also be associated with cancer. In conjunction with evolutionary development of proteases, regulators for proteases have also been developed. These anti-regulators of cellular serine protease are known as serpin. Selective serpins which are thought to be correlated with progression or remission of selected cancers have been selected for the critical reviews so that they can be used for diagnosis and therapy in cancer.

BBI has been widely studied in cancer research for 30 years. BBI treatment in the animal model of oral carcinoma showed a decrease in the number of tumors and diminution of invasiveness compared with the control group [19]. Dietary addition of BBI reduced the incidence of colon adenocarcinoma in both the induced colon carcinoma animal model and in

animals genetically susceptible to spontaneous colon cancers [20,21]. Currently, it has been reported that the anti-carcinogenic effect of BBI derives from its intrinsic ability as a serine protease inhibitor. Chemically inactivated BBI has no significant effect on cancerous cell proliferation [22]. Every isoflavone and protease inhibitor component of soy can suppress prostate carcinogenesis in the rat; however, a combination of both may bemore effective [23]. BBI suppresses activity of membrane-type serine protease 1 (MT-SP1), which plays a significant role in cancer invasion and metastasis [24]. BBI also inhibited the level of c-fos message and was able to decrease c-fos gene expression [25].

Seeds have received increasing attention in recent years because of its nutrition with health-stimulating properties and medicinal effects as they are the storing organs for proteins/peptides [26]. It is not easy to separate edible and medicinal seeds because many of the common edible species that are beneficial in the prevention and treatment of various human diseases. Seeds are rich in protein content. The amount of crude protein in seeds is ranked below most animal meats but above most other foods, such as milk, vegetables and fruits [27]. The high protein content of different medicinally edible seeds makes them a potential source of various drugs for different human life style diseases [28].

The genus *Mucuna* belongs to the climbing legumes (vines and shrubs) of family Fabaceae. *Mucuna* is known to carry approximately 100 species which are used to treat diseases as Ayurvedic medicines from so many decades, of which *Mucuna pruriens* are extensively studied and well documented. Varities of *Mucuna pruriens* include *Mucuna pruriens var. pruriens*, *Mucuna pruriens var. utilis*, *Mucuna pruriens var. hirsute*, *Mucuna pruriens var. sericophylla*, and *Mucuna pruriens var. thekkadiensis* which are currently being utilized for active research [29]. Among them, *Mucuna pruriens*, commonly known as velvet bean or cowitch or kalonch in India, is an important underutilized legume which carries so many biological activities due to the presence of aromatic chemical compounds present. This plant have been studied and identified due the presence of high content of L-3, 4- dihydroxy phenyl alanine (L-DOPA) which is enormously consumed by pharmaceutical industries which is immensely used in the treatment of Parkinsonism disease because of containing an unusual non protein amino acids. Furthermore, Several studies also showed that seeds of *M. pruriens* have numerous pharmacological applications including astringent, laxative, astringent, laxative, anthelmintic, aphrodisiac, alexipharmic and tonic [30,31]. Previous studies on *Mucuna pruriens*

have shown successful isolation of various phytochemical compounds as well as some protease inhibitors such as amylase inhibitors and other pharmaceuticals [32,33]. Hence, no work have carried out on bioactive peptides/protease inhibitors from this plant, therefore, the research work was undertaken to study the protease inhibitors and their importance in human life with the following objectives.

Objectives:

- Screening of Proteins and peptides/protease inhibitors from the seeds of different species of *Mucuna.*
- Isolation and Purification of protease inhibitors from the seeds of *Mucuna pruriens.*
- Characterization of purified protease inhibitor for its Physico-Chemical and biological properties.

1. Screening of proteins and peptides/protease inhibitors from the seeds a verities of Mucuna pruriens.

1.1. Collection of Seeds

Seeds of *Mucuna pruriens* were collected from IIHR-Bangalore. The seeds were stored at 4°C for experimental use.

1.2. Preparation of Acetone powder and Butanol powder

Mucuna seed were used for screened out the presence of trypsin inhibitor. The seeds of *Mucuna pruriens* were soaked and dehulled. The dehulled seeds were defatted with chilled acetone and butanol (10% each) and acetone and butanol powder were prepared. The cakes obtained were collected, air dried, powdered separately, and stored at 4°C for further analysis.

1.3. Differential extraction of proteins/protease inhibitors

The 10% crude protein extracts of the acetone and butanol powder were prepared using different solvents like Water; 0.05M sodium acetate buffer, pH 5.0; 0.05M sodium phosphate buffer, pH 7.0; 1% NaCl; 0.05M Tris-HCl buffer, pH 8.0; 0.1N HCl and 0.1N NaOH by stirring over magnetic stirrer for 2 hrs at 4°C and then Centrifuged at 10,000 rpm for 30 min. at 4°C. The supernatant was collected and stored in 0-4°C for further analysis.

1.4. Determination of total protein and protease inhibitor activity

The total proteins in the crude extracts of the seeds of *Mucuna pruriens* were determined by the method of Lowry et al. 1951 [34], using bovine serum albumin (BSA) as standard. The protein content in all the differential extracts of *Mucuna pruriens* seeds shows considerable variations from 15% - 32%. The protein content for in all the differential extractions are shown (Fig. 1). The trypsin assay was performed with BAPNA as substrate according to the method of Shibata et al. 1986 [35]. Trypsin was dissolved in 0.001N HCl containing 20mM at a concentration of 200µg/ml. The 40mg BAPNA was dissolved in 1ml DMSO and according to usage, the DMSO solution was then dissolved in 0.2M Tris-HCl pH 8.2 containing 0.2M $CaCl_2$ and rest with distilled water. The assay mixture contains 500µl of trypsin, 450 µl suitable buffer and 1.25ml BAPNA incubated for 15 min. at room temperature and the reaction was terminated by adding 0.4ml of 30% Acetic acid. The reaction absorbance for color development was read at 410nm. 1 Trypsin unit (TU) is defined as increase in the OD of 0.01 at 410 nm. Trypsin inhibitory assay was performed according to the reported method of Shibata et al., (1986) [35] using BAPNA as substrate. The assay contains 0.5ml of Trypsin, 400µl of appropriate buffer and 100µl of Trypsin inhibitor, incubated for 15 min. at room temperature. The 1.25ml BAPNA was added and again incubated for 10 min. at room temperature. The reaction was terminated by added 0.4ml of 30% Acetic acid. The OD was taken at 410nm for color development against blank (Fig. 1). Trypsin inhibitor units: One Trypsin inhibitor unit (TIU) is defined as decrease in the OD by 0.01 at 410 nm. The trypsin inhibitory ctivity ranges from 9.2 – 66 TIU/mg of protein (Fig. 1). The Trypsin inhibitory activity in all the differential extractions are shown (Fig. 1).

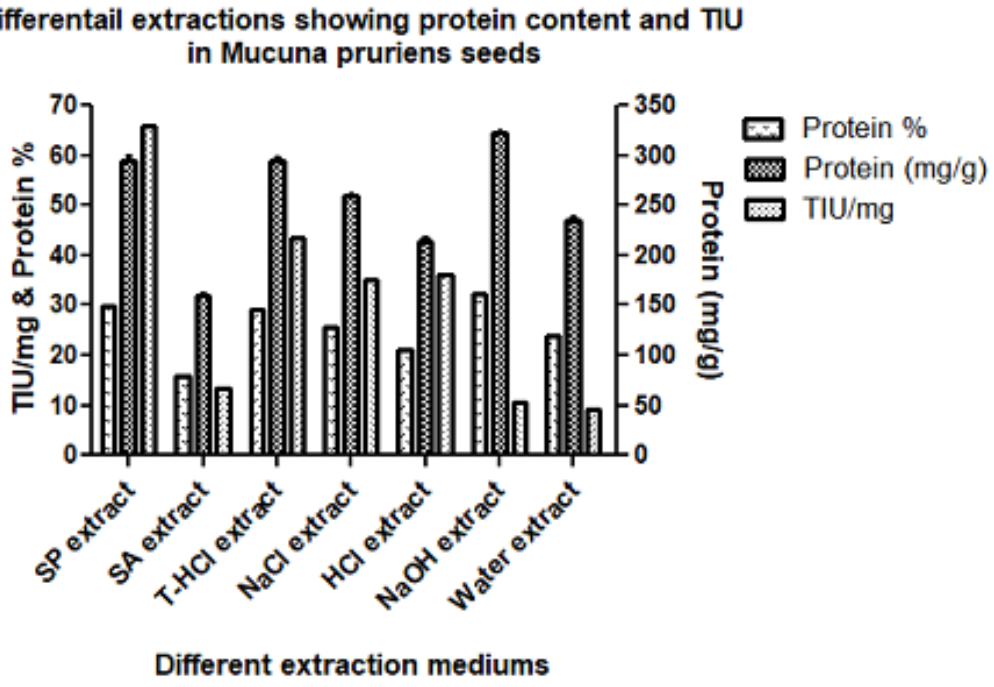

Fig.1: Differential extraction mediums showing the protein content, protein percentage and Trypsin inhibitory activity (TIU) in *Mucuna pruriens* seeds

1.5. Electrophoretic analysis

A discontinuous cationic slab gel electrophoresis was carried out essentially according to the method of Reisfield et al., (1962) [36], and is composed of 10% separating and 5% stacking gel system. The gel was removed carefully and kept for protein staining (0.02% Coomassie brilliant blue R-250) and destained with methanol and acetic acid solution. The appearance of blue bands indicated the presence of proteins. Reverse Zymography was done for visualization of trypsin inhibitor on PAGE according to (Felicioli et al., 1997) [37], by adding 1 ml of 1% gelatin solution to the resolving gel. After electrophoresis, native gel was washed with distilled water and incubated in trypsin (100 µg/ml solution for 30 min, and stained for inhibitory activity with the solution comprising of 0.1% Coomassie brilliant blue R-250 (w/v) in 10% Acetic acid and 20% Isopropanol for 1 hr. The gel was destained in 10% acetic acid and 25% isopropanol/methanol solution overnight. The appearance of dark blue bands indicates the presence of trypsin inhibitor. The gels were stored in 7.5 % acetic acid). Electrophoresis analysis revealed the presence of four major and two minor protein bands in Sodium phosphate, Tris HCl, Sodium chloride and sodium acetate extracts followed by 6 major protein bands in Water extract,

one major and one minor protein bands in HCl extract and a large diverse protein band in sodium hydroxide extract. Reverse Zymography study revealed the presence of four inhibitory bands in Sodium chloride extract and Tris HCl extract, three inhibitory bands in Water extract and Sodium acetate extract, two inhibitory bands Sodium phosphate extract, One inhibitory band in both HCl and sodium hydroxide extracts (Fig. 2a & 2b). On this basis of single inhibitory protein and trypsin inhibitor bands, the Hydrochloride (HCl) extract was used for further study.

(a): Protein staining **(b): Protease inhibitor staining**

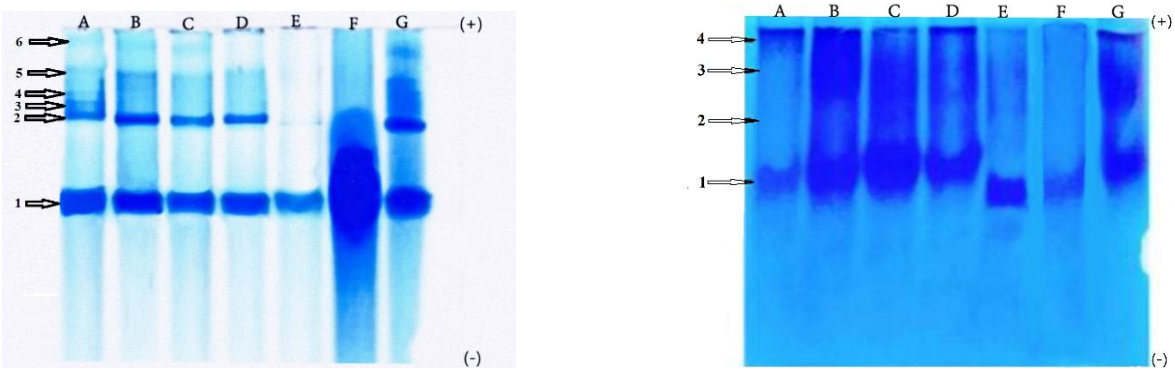

Fig. 2a: Native PAGE profile of *Mucuna pruriens* seeds in differential extraction mediums. Fig. 2b: Reverse zymography PAGE pattern of Serine protease inhibitor from *Mucuna pruriens* seeds. (Lanes A: Sodium phosphate extract, B: Sodium acetate extract, C: Tris-HCl extract, D: Sodium Chloride extract, E: Hydrochloric acid (HCl) extract, F: Sodium hydroxide extract, and G: Water extract)

1.6. Antibacterial activity of isolated serine protease inhibitor extracts

The antibacterial activity of differential extracts of *Mucuna pruriens* seeds were performed by agar well diffusion method as described by [38] against multidrug-resistant pathogenic bacteria, *Salmonella typhi and Enterococcus faecalis*. The Nutrient agar (10 gms) was dissolved in double distilled water (250 ml) and shaken smoothly. The mixture was boiled in hot air own, plugged with cotton and then autoclaved for 20 minutes. The inoculums were spread on the Muller Hinton agar medium and 7 wells with 5 mm diameter were made. The differential crude inhibitor extracts (200 µg) of *Mucuna pruriens* seeds were added and streptomycin (50 µg) antibiotic was taken as standard. These plates were kept for incubation at 37°C for 24 hours. The results were examined for growth and the presence or absence of inhibition zone around the well. Water; sodium phosphate; Tris-HCl and HCl extracts showed maximum zones of inhibition and sodium acetate and sodium chloride extracts observed little inhibition against *Salmonella typhi* and *E facealis.*

2. Screening of Proteins and peptides/protease inhibitors from the seeds of different species of *Mucuna.*

2.1. Collection of Seeds

Seeds of seven different species/varieties of *Mucuna pruriens* were collected from different parts of South India. *Mucuna pruriens, Mucuna utilis and Mucuna pruriens* V2, were collected from IIHR-Bangalore. *Mucuna pruriens* V1 was collected from Somvarpete and *Mucuna hirsute* was collected from Madikeri taluks of District Kodagu, Karnataka. *Mucuna monosperma* and *Mucuna gigantea* were collected from TGBRI-Kasargod Kerala. All the seeds were stored at 4°C for experimental use.

2.2. Preparation of crude inhibitor extractions

The 10% protease inhibitor extracts of the soaked and dehulled seeds were prepared in Sodium Phosphate buffer pH 7, by the method of Wetter *et al*., 1957 as described earlier. The supernatant collected were stored at 4°C for further analysis.

2.3. Determination of total protein protease inhibitory activity

The total protein was determined according to the method of (Lowry *et al*., 1951a) using bovine serum albumin (BSA) as standard. The variation in the total storage soluble proteins showed significant differences between different species/varieties of *Mucuna* seeds and was found to be ranged between 18% - 29% (Table 1). Among the different species/varieties analyzed, the protein content of *Mucuna pruriens* (27.435 mg/ml) and *Mucuna utilis* (29.025 mg/ml) were higher than other species/varieties like *Mucuna pruriens V1* (18.753 mg/ml), *Mucuna hirsute* (23.889 mg/ml), *Mucuna pruriens V2* (21.707 mg/ml), *Mucuna gigantae* (24.344 mg/ml), and *Mucuna monosperma* (26.980 mg/ml) (Table 1). The trypsin activity and trypsin inhibitory activity were determined according to the method as described earlier (Shibata *et al*., 1986a) [35]. Seeds of different species/varieties of *Mucuna* contain considerable variation in trypsin inhibitory activity ranging between from 9.78 – 18.25 TIU/mg of protein (Table 1). The maximum trypsin inhibitory activity was found in *Mucuna pruriens* (18.25 TIU/mg), and minimum in *Mucuna pruriens* V1 (9.78 TIU/mg). The trypsin inhibitory activity of other species/varieties of *Mucuna* showed variations such as: *Mucuna pruriens* V2 (13.02 TIU/mg),

Mucuna utilis (16.67 TIU/mg), *Mucuna hirsute* (15.93 TIU/mg), *Mucuna gigantae* (11.98 TIU/mg), and *Mucuna monosperma* (15.27 TIU/mg).

Table-1: Total protein content and total trypsin inhibitory units in different *Mucuna* species/varieties

No	Sample	Total protein (mg/ml)	TIA (TIU/mg)
1	*Mucuna pruriens V2*	27.43	18.25
2	*Mucuna utilis*	29.02	16.67
3	*Mucuna hirsute*	23.88	15.93
4	*Mucuna gigantea*	24.34	11.98
5	*Mucuna monosperma*	26.98	15.27
6	*Mucuna pruriens V1*	18.75	9.78
7	*Mucuna pruriens*	21.70	13.02

2.4. Electrophoretic analysis

A discontinuous cationic slab gel electrophoresis contained 10% separating gel, was carried out essentially according to the method of (Reisfeld *et al*., 1962a) as described earlier. Reverse Zymography was done for visualization of trypsin inhibitor on native PAGE according to method of (Felicioli *et al*., 1997), as described earlier. The gels were stored in 7.5 % acetic acid). Electrophoretic analysis of the crude extracts of different species/varieties of *Mucuna* seeds on native PAGE revealed the presence of multiple protein bands (Fig 3a). *Mucuna pruriens* V2 (6 major and 8 minor bands) and *Mucuna utilis* (8 major and 8 minor bands) were higher major bands than other species/varieties like *Mucuna pruriens* (4 major and 3 minor bands), *Mucuna hirsute* (7 major and 9 minor bands), *Mucuna pruriens* V1 (1 major and 1 minor bands), *Mucuna gigantae* (1 major and 2 minor bands), and *Mucuna monosperma* (9 major and 6 minor bands). Protein electrophoretic profiles of different *Mucuna* species/varieties showed almost identical patterns in both major and minor protein bands, which infers the stability of seed storage proteins within these species/varieties. However, considerable variation of protein patterns was observed among the seeds of different varieties of *Mucuna* such as *Mucuna pruriens, Mucuna pruriens V1* and *Mucuna pruriens V2*. This could be correlated to the different geographical regions of the south India.

Electrophoretic analysis of trypsin inhibitors revealed the presence of three to nine isoinhibitors in the seeds of different species/varieties of *Mucuna* (Fig. 3b). The *Mucuna hirsute, Mucuna pruriens V2, Mucuna gigantae, Mucuna monosperma and Mucuna utilis* contained a maximum of nine isoinhibitors of trypsin while *Mucuna Pruriens* and *Mucuna pruriens* V1 contains five and three trypsin isoinhibitors respectively.

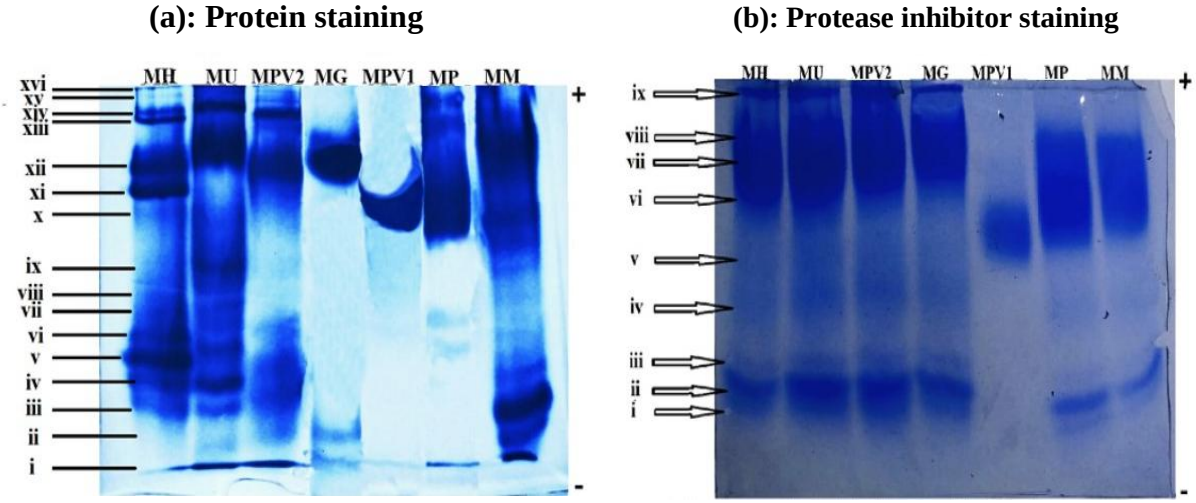

Fig. 3: Electrophoretic analysis of seeds extracts of *Mucuna* species (a) native PAGE for proteins staining and (b) reverse zymography PAGE for trypsin inhibitor staining. (Lanes MH: *Mucuna hirsute*, MU: *Mucuna utilis*, MPV2: *Mucuna pruriens variety 2*, MG: *Mucuna gigantae*, MPVI: *Mucuna pruriens* variety 1, MP: *Mucuna pruriens*, and MM: *Mucuna monosperma*)

3. Isolation and Purification of protease inhibitors from the seeds of *Mucuna pruriens.*

3.1. Isolation and purification of protease inhibitor

The *Mucuna pruriens* seeds were soaked, dehulled and 10% butanol powder was prepared according to the method of Wetter 1957 [39]. The dehulled seeds were grinded with chilled butanol followed by filtration under suction pump. The cake obtained was collected, dried, powdered and stored at 4°C until further use. The 10% crude trypsin inhibitor extract was prepared in 0.1N HCl on a magnetic stirrer at 4°C for 1 hr. followed by centrifugation at 10,000 rpm for 30min at 4°C. The supernatant was collected was used for determining the protein content and Trypsin inhibitory activity. Protein estimation trypsin inhibitory activity assays were performed as described earlier. The supernatant was collected and brought to different saturation levels as 0-25%, 25 – 50% and 50-90% by gradual addition of ammonium sulfate $(NH_4)_2SO_4$ with constant stirring for 1.3 hrs and kept in refrigerator over-night for each fractionation process

[40]. The stored fractions were employed for centrifugation at 10,000 × g for 30 min and pellets was collected for each fraction. The pellets obtained was dissolved in 25mM Sodium Acetate buffer, pH 5.7 and dialyzed against the same at 4°C with different concentrations. The dialysis membrane was activated by boiling in 25mM Sodium Acetate buffer, pH 5.7 for about 10-15min. and rinsed with distilled water and allowed it to cool at room temperature and stored at 4°C in 50% ethanol.

CM-Cellulose powder (10g) was dissolved in 500ml of distilled water for about 24 hr at room temperature. Then, 200 ml of 0.1N HCl solution was added to the cation-exchanger by stirring it continuously and kept for 30min. at room temperature, followed by decanted the HCl solution carefully. The matrix was activated and then was equilibrated with the addition of 200ml of 25mM Sodium Acetate buffer, pH 5.7.

The equilibrated matrix was poured into the glass column (2.3×7.5 cm) and allowed it to settle. The column was pre-equilibrated overnight with 0.025mM Sodium Acetate buffer, pH 5.7. The column was washed with running buffer (0.025mM Sodium Acetate buffer, pH 5.7) at a flow rate of 60 ml/hr with a fraction volume of 10 ml. After the equilibration, the dialyzed sample after obtained from ASF (25-50%) with our desired inhibitor was loaded on to the M-Cellulose column and washed with three bed volumes of 0.025mM Sodium Acetate buffer, pH 5.7. The unbound fractions were collected and bound proteins were eluted by stepwise elution with 0.1M, 0.3M and 0.5M NaCl (0.1-0.5M NaCl) in 25mM Sodium Acetate buffer, pH 5.7. The unbound and bound fractions were assayed for protein content and trypsin inhibitory assay using BAPNA as substrate (Fig. 4a). The bound CM-cellulose fraction containing trypsin inhibitor activity were pooled and concentrated by using Centricon tubes of 5kDa size.

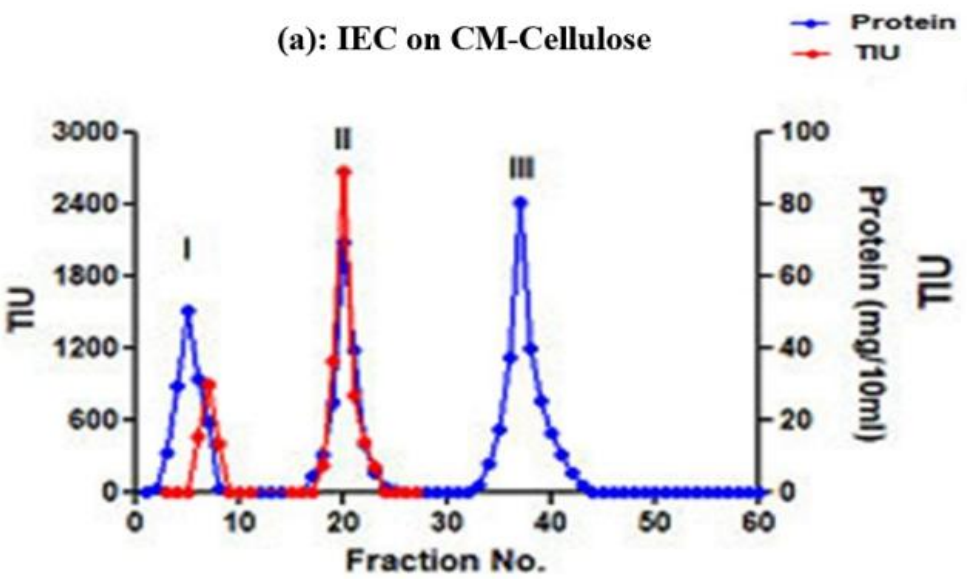

Fig. 4a: Separation of protease inhibitors during ion exchange chromatography

The concentrated CM-cellulose fraction was subjected to Sephadex G-75 gel filtration chromatography. Sephadex G-75 powder was suspended in 500ml of distilled water and allowed to swell for about 5 hrs on water bath and then kept at room temperature until it cooled down. The water and unwanted fines were decanted from the gel and pre-equilibrated with 25mM Sodium Acetate buffer, pH 5.7. The opening of the column was tight fixed and glass mesh was inserted onto the opening of the column. The slurry was carefully poured into glass column (1.5 × 150cm) so as to avoid air bubbles and allowed to settle under gravity. The Sephadex G-75 column was equilibrated for about 2 days with one and half bed volumes 25mM Sodium Acetate buffer, pH 5.7 with an elution rate of 2ml/12 minutes. The concentrated CM-Cellulose fraction II was loaded on to the column. The fractions with 25mM Sodium Acetate buffer, pH 5.7 were collected. All the fractions were analyzed for protein content trypsin inhibitory activity (Fig. 4b).

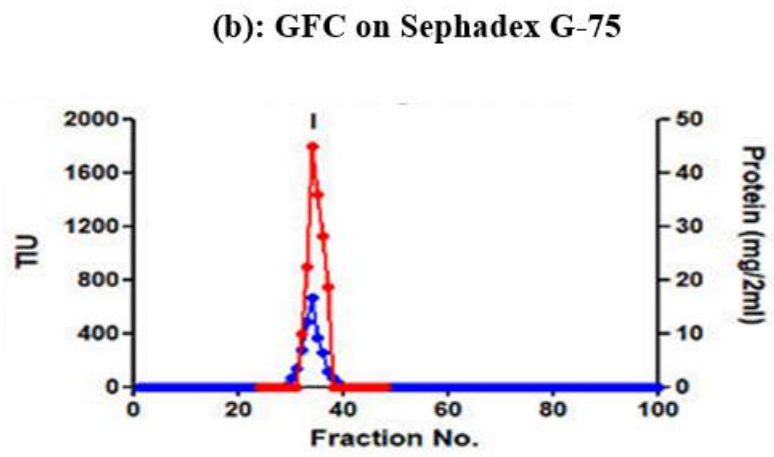

Fig. 4b: Separation of protease inhibitors during gel filtration chromatography

The fractions containing trypsin inhibitor activity were pooled and concentrated by Centricon tubes of 5kDa. The trypsin inhibitor was purified to 55.702 fold with a specific inhibitory activity of 202.31 and 6.104 % recovery (Table 2).

3.2. Criteria of purity

The Centricon tube concentrated fraction of sephadex G-75 gel filtration chromatography was further subjected to preparative gel electrophoresis. 10% Non-denaturing preparative cationic PAGE was performed at pH 4.3 (10% T, 5% C) according to the method of Reisfield *et al.* (1962) [41]. After the electrophoresis, the electrophoretic bands containing trypsin inhibitor activity were sliced, transferred and macerated in a test tube using glass rod at 4^{o}C. The solution was subjected to centrifugation at 5000 rpm for 10 minutes at 4^{o}C to remove gel particles. The supernatant obtained was purified trypsin inhibitor.

Table 2: Purification profile of Cationic serine protease inhibitor from *Mucuna pruriens* seeds

3.3. Electrophoretic study

10% Non-denaturing native cationic PAGE was performed at pH 4.3 (10% T, 5% C) was done acc. to the method of Reisfield *et al.*, (1962) by using the vertical electrophoretic chamber

Purification steps	Protein (mg)	TIU	Specific Inhibitory activity (TIU/mg)	% Yield	Fold of purification
Crude	4735.469	17200	3.632	100	1
ASF 25-50%	571.32	7200	10.725	41.86	2.952
CM Cellulose Chromatography	170.49	5430	31.849	31.56	8.768
Sephadex G-75 Chromatography	50.07	3680	73.497	21.39	20.235
Preparative PAGE (MPTI)	5.19	1050	202.31	6.104	55.702

[41].The acrylamide and bis-acrylamide solutions were prepared in the ratio of (29.2:0.8). The slab resolving gel 10% was prepared by Acetic acid-KOH buffer pH 4.3 and 5% stacking gel by Acetic acid-KOH buffer pH 6.8 with double distilled water, Riboflavin (4%) and 0.28% APS and appropriate volume of TEMED. The resolving gel was kept in sunlight for 30min. for photo-polymerization. The electrode chambers were filled with electrode buffer of pH 4.5 prepared by (3.12g βalanine, 0.8ml Acetic acid and diluted up to 600ml water). The sample buffer of pH 4 was prepared by 0.006M buffer, 20% Sucrose and a pinch of methyl green made up to 10ml by added water. The samples were diluted with sample buffer in the ratio of (10:2) and loaded onto each sample well and subjected to electrophoresis at (4ºC) by applying a current of 50mA for the first 30 min. and then 90mA for 2-2.5 hr. After the run, the gels were removed and stained for proteins using 0.02% Coomassie brilliant blue G-250 (w/v) in 3.5% (w/v) Perchloric acid for 1 hr. and washed several times with water. The gel was destained in distilled water overnight. The appearance of blue color bands confirms the presence of proteins (Fig. 5a)

12.5% Non-denaturing preparative SDS PAGE was performed at pH 8.3 (10% T, 5% C) was done acc. to the method of [42].The acrylamide and bis-acrylamide solutions were prepared

in the ratio of (29.2:0.8). The slab gel (12% resolving and 5% stacking gel) was prepared and the gel was placed into the vertical electrophoretic chamber. The resolving gel was prepared by 1.5M Tris-HCl pH 8.08 and stacking gel by 0.5M Tris-HCl pH 6.8. The 10% SDS and APS with an appropriate volume of TEMED were used. The electrode chambers were filled with electrode buffer of pH 8.3 prepared by (25mM Tris-HCl + 192mM glycine and 10% SDS). The sample buffer was prepared by 0.5M Tris pH 6.8, 3ml of glycerol, 1.2mg of Bromophenol blue and 10% SDS made up to 10ml by added water. The samples were diluted with sample buffer in the ratio of (10:2) and loaded into each sample well and subjected for electrophoresis at (4ºC) by applying a current of 50mA for the first 30 min. and then 90mA for 2-2.5 hr. After the run, the gels were washed with 2.5% Triton X-100 in the TB sol. (2×30min.) to remove the SDS and then washed with water several times. The gels were kept for protein staining using 0.1% Coomassie brilliant blue R-250 (w/v) in 10% Acetic acid and 20% Isopropanol for 1 hr. and after incubation, washed several times with water. The gel then was destained in 10% Acetic acid and 25 % methanol/ Isopropanol overnight and stored in 7.5 % Acetic acid. The appearance of blue color bands confirms the presence of proteins. The electrophoretic analysis revealed single protein band corresponding to the single trypsin inhibitory band at all purifications stages. The presence of single protein band of the purified MPTI is shown in Fig. 6b (I). The 12.5% SDS reducing PAGE was also performed as described with some modifications, by adding 0.5ml βmercaptoethanol in the sample buffer, boiled the samples at 100°C for 5 min. and cooled before use. The SDS-PAGE of MPTI is shown in Fig. 6a.

Reverse Zymography was done for visualization of trypsin inhibitor in polyacrylamide gel was performed according to Felicioli *et al.,* (1997) with little modifications, by adding 1ml of 1% gelatin solution to the resolving gel [37]. After electrophoresis, the native gel was washed with water and then incubated in Trypsin (200µg/ml solution for 30 min. and washed for 3 × 1min. then stained for inhibitory staining with 0.1% Coomassie brilliant blue R-250 (w/v) in 10% Acetic acid and 20% Isopropanol for 1 hr. and washed several times with water and kept in a destaining solution comprising 10% Acetic acid and 25% Isopropanol/Methanol overnight. The appearance of dark blue bands indicates the presence of Trypsin inhibitor (Fig. 5b). The gels were stored in 7.5 % acetic acid). The presence of single trypsin inhibitory band of the purified MPTI is shown in Fig. 6b (II).

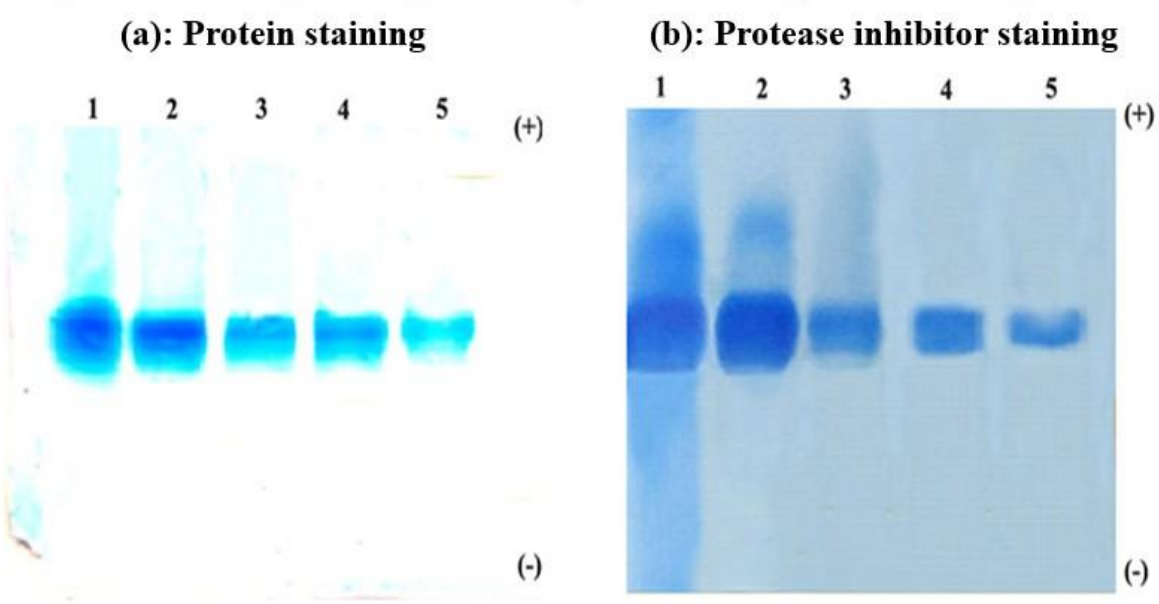

Fig. 5: Native PAGE pattern of proteins (a) and protease inhibitor (b) profile of *Mucuna pruriens* seeds (Lanes 1: Crude inhibitor extract, 2: Ammonium sulfate fraction (25-50%), 3: CM-Cellulose fraction-II, 4: Sephadex G-75 fraction, and 5: Preparative PAGE fraction)

4. Characterization of purified protease inhibitor for its Physico-Chemical and biological properties.

4.1. Determination of Molecular mass

SDS-PAGE was carried out in 12.5% polyacrylamide gel (12.5% T, 5% C) in both the presence and absence of β-mercaptoethanol [31]. After the electrophoretic run, the gel was removed, stained and destained as described above. The non-reducing band 37 kDa (TINR-37) was sliced, transferred and macerated in glass homogenizer at 4oC followed by centrifugation at 5000 rpm for 10 minutes. The supernatant obtained was subjected to native PAGE for both protein and inhibitor staining. The SDS PAGE was also conducted both in presence and absence of β-mercaptoethanol, as described above. It was found that the purified protease inhibitor exhibited a molecular weight of 37 kDa (Fig. 6a).

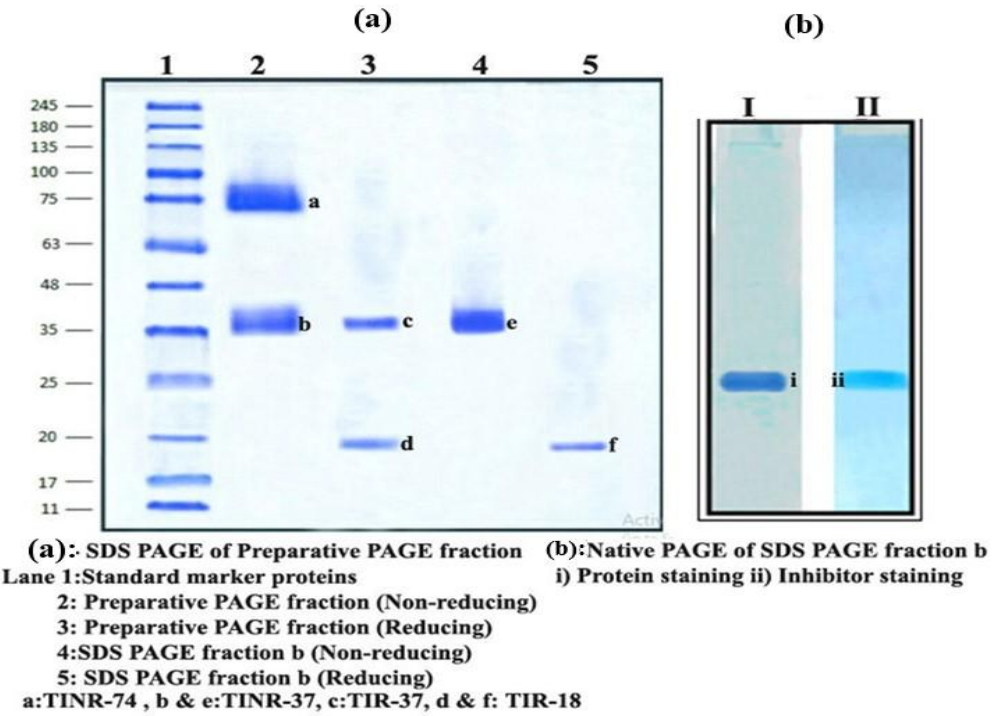

Fig. 6: (a)-SDS PAGE and (b)-Native PAGE of MPTI

4.2. Identification and Characterization of MPTI by UPLC/ESI-QTOF-MS

The MPTI bands (PINR74, PINR37, PIR37 and PIR18) were eluted from SDS PAGE separately with buffer, mixed with an equal volume of 1 M $CaCl_2$ and incubated at room temperature for 10 min [43]. The mixture was centrifuged at 10,000×g for 10 min, and proteins in the supernatant were collected and filtered using Whatmann filter paper no. 1. The filtered samples were analyzed in an ultra-performance liquid chromatography system (Acquity UPLC from Waters Corporation, USA) coupled to mass spectrometer with ESI-QT of-MS capabilities (Synapt G2-Si from Waters Corporation, USA). The separations were carried out using an ethylene bridged hybrid, C4, 2.1 mm x 50 mm, 1.7 µm column in presence of mobile phases containing 0.1% formic acid in water and 0.1% formic acid in acetonitrile using a focused gradient with a flow rate of 0.4 mL/min. Mass spectrometry conditions were optimized with capillary voltage of 3.0 kV, cone voltage of 40 V with source and desolvation temperatures of 120°C and 350° C, respectively. The LCMS analysis also revealed that the purified trypsin inhibitor exhibited a molecular weight of 37.39 kDa (Fig. 7).

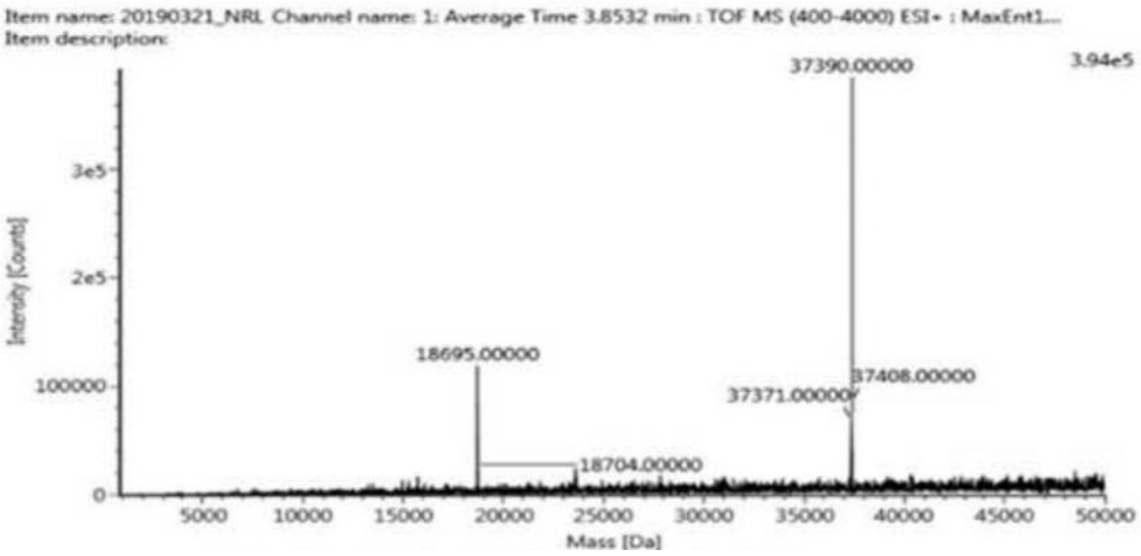

Fig. 7: UPLC/ESI-QTOF-MS analysis of TINR-37 (MPTI)

4.3. N-terminal sequencing by ESI/MALDI-QTOF-MS

The TINR-37 was subjected to N-terminal sequencing using ESI/MALDI-QTOF-MS (Bruker Corporation, USA) [43], at the division of Molecular-Biophysics unit, Indian Institute of Science, Bangalore. The N-analysis showed 100% sequence similarity with the N-terminal end of most of the BBI type of protease inhibitors.

4.4. Determination of Kinetic studies and inhibitor constant (Ki)

The Km and Vmax of trypsin for BAPNA in the presence of MPTI was determined from Lineweaver-Burk plot and the inhibition constant was calculated from secondary plots of competitive inhibition [44]. The enzyme to the inhibitor ratio was determined for MPTI against bovine trypsin. The residual inhibitory activities were determined using BAPNA as substrate as described earlier. The increased Km in presence of MPTI indicated that the MPTI acts as competitive inhibitor (Fig. 8). The inhibitor constant was found to be 1.3×10^{-6}M.

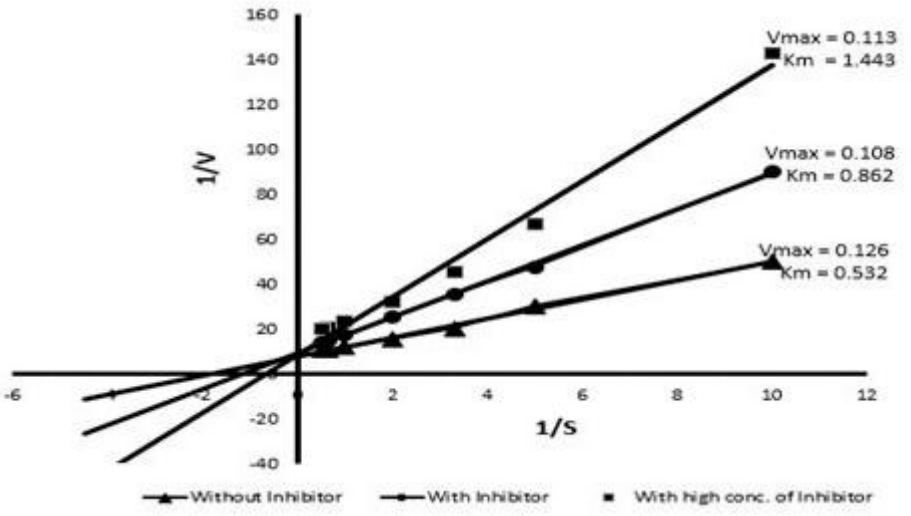

Fig. 8: Determination of Km and Vmax of trypsin in the presence of MPTI

4.5. Absorption spectroscopy

In order to determine the presence of protein or peptides, the Absorption spectroscopy was performed (Fig. 9). This analysis revealed that MPTI is proteinaceous in nature.

4.6. Circular Dichorism

CD analysis was done for determining the presence of Secondary structure in the purified inhibitor. It was found that the purified MPTI exhibited well secondary structure (Fig. 10).

4.7. Florescence spectroscopy

In order to verify the overall territory structure of purified protein, intrinsic florescence was carried out (Fig. 11), which revealed well defined tertiary structure of the purified MPTI.

4.8. Effect of pH on MPTI tertiary structure using ANS binding

After analysis of tertiary structure of MPTI, we intended to determine the changes in tertiary structure of MPTI by using the range of pH values using fluorescence spectroscopy. Intrinsic fluorescence is used to observe the structural changes in hydrophobic amino acid environment i.e. structural and conformational changes in the tryptophan residues. This analysis revealed that MPI is structurally stable in the acidic conditions and lost its structural stability at highly alkaline conditions (Fig. 12).

4.9. Thermal denaturation study

The protein was analyzed for the thermal stability using range of temperature from 25^{o}C to 90^{o}C. The protein was thermostable around 74^{o}C and thereafter, the unfolding transition appeared and was completely denatured around temperature of 88^{o}C.

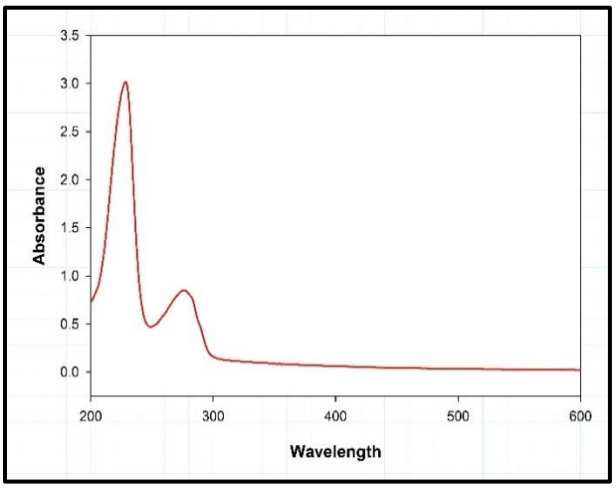

Fig. 9: Absorption spectroscopy spectrum of MPTI

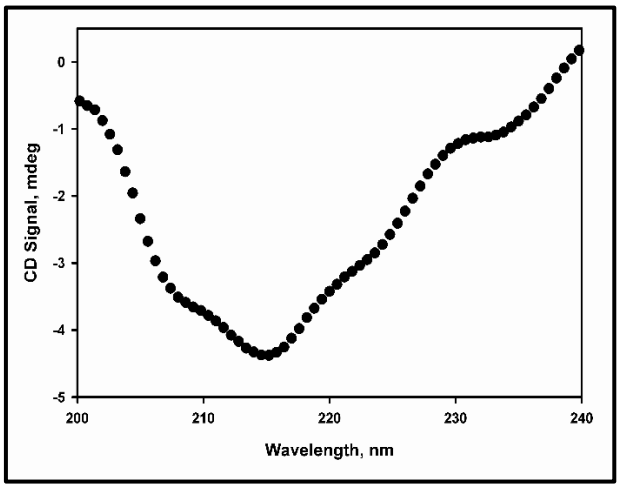

Fig. 10: Far-UV CD Spectra of purified MPTI protein

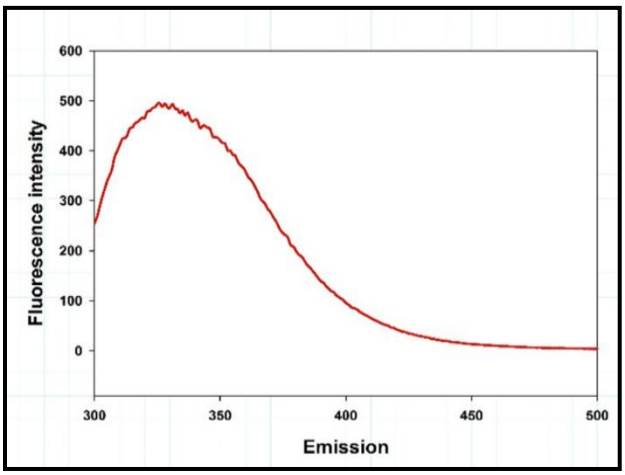

Fig. 11: Florescence spectroscopy spectrum of MPTI

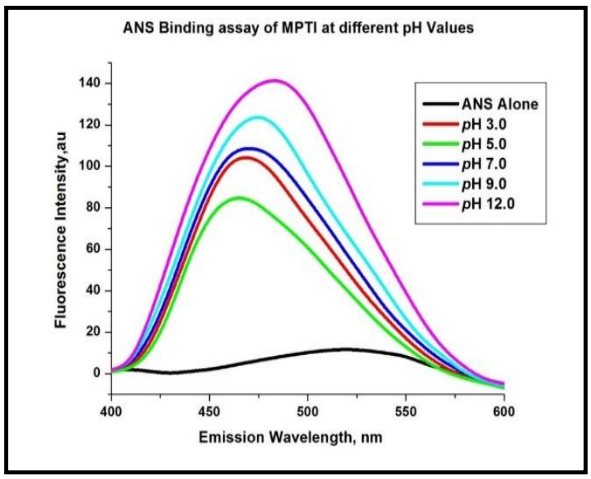

Fig. 12: ANS-binding analysis of MPTI

4.10. Effect of Temperature on functional activity of the MPTI

To determine the optimum temperature of inhibitor, the activity of the purified inhibitor with enzyme was estimated with varying temperatures from 4°- 80°C. Again the protein was incubated for 30min. at varying temperatures and enzyme was added to check the stability of the protein at particular temperature after the samples were cooled down. The all assays were evaluat1ed for Trypsin inhibitory activity using BAPNA as substrate according to the method of Kakade et al. [45]. The purified MPTI was functionally stable between 4°- 70°C and its optimum temperature was found to be 30°C.

4.11. Effect of pH on functional activity of the MPTI

To determine functional stability and optimum pH of the purified MPTI, the activity of the purified inhibitor with enzyme was treated with different buffers with ranging from pH 2 to 12 including 0.1M glycine HCl buffer (pH 2-3), 0.1M sodium acetate buffer (pH 4-5.5), 0.1M sodium phosphate buffer (pH 6.5-7), 0.1M Tris HCl buffer (pH 8-9), 0.1M glycine-NaOH buffer (pH 10-11) and 0.1 M potassium chloride buffer (pH 12). After incubation, sample was assayed for Trypsin inhibitory activity using BAPNA as substrate according to the method of Kakade et al. [45]. Again the inhibitor alone was treated with these above buffers for 30 min. with varying pH ranging from 2 to 12 and after, other ingredients were added for determining the pH stability of the protein. The functional activity optimum of the purified MPTI was found maximum at pH 5, hence the called the optimas pH and was found to be functionally stable in a wide pH range.

4.12. Effect of metallic ions on functional activity of the MPTI

The stability of the Trypsin inhibitor was tested in different metal ions by the standard procedure to identify potential inhibitors or activators. The action of different salts of 0.5% and 1% of metallic cations like ($CaCl_2$, $MgCl_2$, $HgCl_2$, $ZnCl_2$, NaCl & $CuSO_4$) was determined by incubating the trypsin inhibitor with above ionic solutions for 30 min separately, followed by addition of other required ingredients. The activity in the absence of any additives was taken as 100%. The all assays were evaluated for Trypsin inhibitory activity using BAPNA as substrate according to the method of Kakade et al. [45]. It was analysed that the purified MPTI retained its maximum activity in the presence of most of the metallic ions.

4.13. Effect of reducing agents on functional activity of the MPTI

The trypsin inhibitor was further analyzed for the ionic, non-ionic detergents and reducing agents such as phenylmethylesulfonyl fluoride (PMSF), tetra- sodium ethylene-diamine-tetra-acetate (EDTA), beta-mercaptoethanol (β-ME) and Sodium dodecyl sulphate (SDS) (25mM and 50mM), by incubating the MTI for 1 h and their residual inhibitory activities were estimated. The all assays were evaluated for Trypsin inhibitory activity using BAPNA as substrate according to the method of Kakade et al. 1969 [45]. It was found that the purified MPTI retained its maximum activity in the presence of Sodium dodecyl sulphate (SDS).

4.14. Anti-inflammatory activity

Hemolytic assay was performed [46], with slight modifications. Goat blood sample (20ml at 0-4°C) was collected from slaughter house. The RBC suspension solution was prepared by centrifugation of blood sample at 3000rpm at 4°C for 5min followed by washing thrice with 0.85% sodium saline. Suspension was made up to 20ml with isosaline (10% v/v). The assay mixture (1ml of different concentrations of MPTI with 2ml of hyposaline and 0.5ml of RBC suspension) was incubated for 30 min and then centrifuged at 1500 rpm for 10 minutes. The supernatant obtained was collected and absorbance was read at 560nm using spectrophotometer (SP 3000-Plus, Optima Tokyo Japan). The control was prepared using hyposaline and RBC suspension. Diclofenac (1mg/ml) was taken as standard drug. It was found that the purified MPTI showed significant anti-inflammatory activity (Fig. 13).

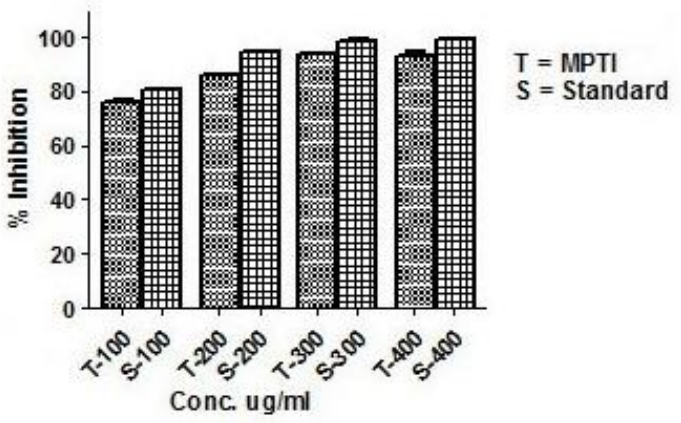

Fig. 13: Anti-inflammatory activity of purified MPTI

4.15. Anti-cancer activity

4.15.1. Cytotoxicity Assay

The anti-cancer property of the MPTI was accessed by Trypan blue assay by the method of [47]; for this, HeLa and Hek 293 cells (1 x 106 cells/well) were seeded in a 24 well plate. After the adhesion, the cells were treated with different concentrations of MPTI (0.0036 - 2000µg) after 24 hours of treatment, the cells were accessed for cytotoxicity by trypan blue assay. It was observed that the purified MPTI showed potent anticancer activity against HeLa and Hek cancer cells with IC50 values of (250.47 µg/ml) and (581.34 µg/ml) respectively (Fig. 14).

4.15.2. Comet assay (Single cell Gel-electrophoresis)

Comet assay was performed as reported by [48] with little modifications. Briefly, HeLa cells treated with MPTI were collected and resuspended in cold phosphate buffer saline. 0.4 ml cell suspension was added to 1.2 ml of 1% low melting agarose and spread uniformly on slides and allowed to solidify at room temperature, followed by incubation in lysis buffer (2% SDS + 0.5M Na_2-EDTA pH >10) for 19 hours at 37˚C. The slides were washed with TAE buffer several times and then subjected to electrophoresis followed by staining in propidium iodide. The excess stain was washed with distilled water. The slides were then analyzed under a fluorescent microscope for comets. The results confirmed the cell death by dosing with purified MPTI (Fig. 15).

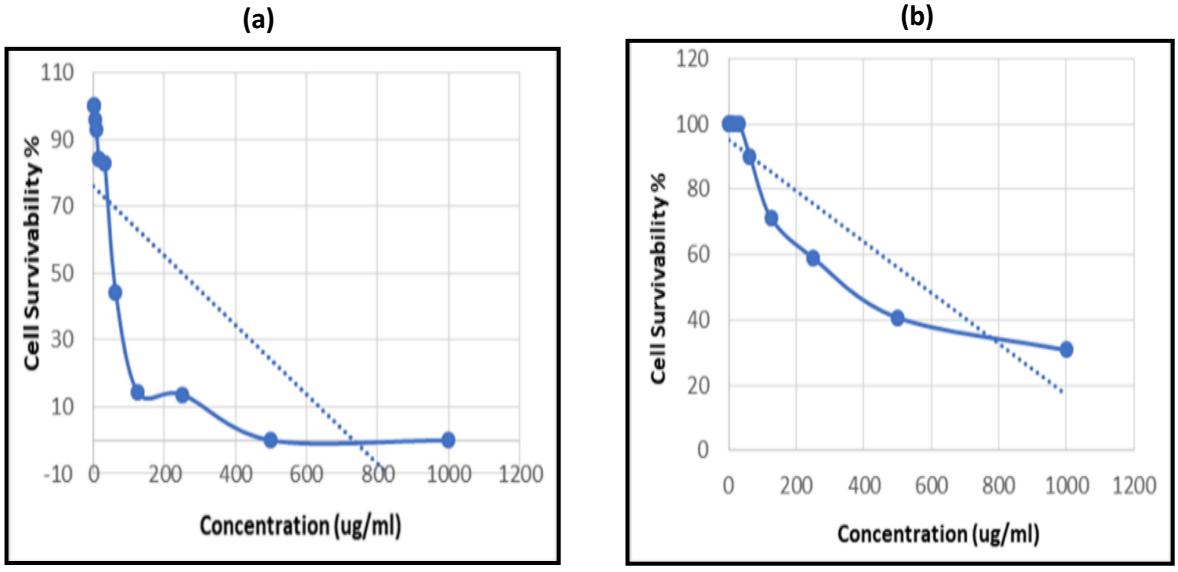

Fig. 14: Cytotoxicity effect of the purified MPTI (a) HeLa cells (b) Hek cells

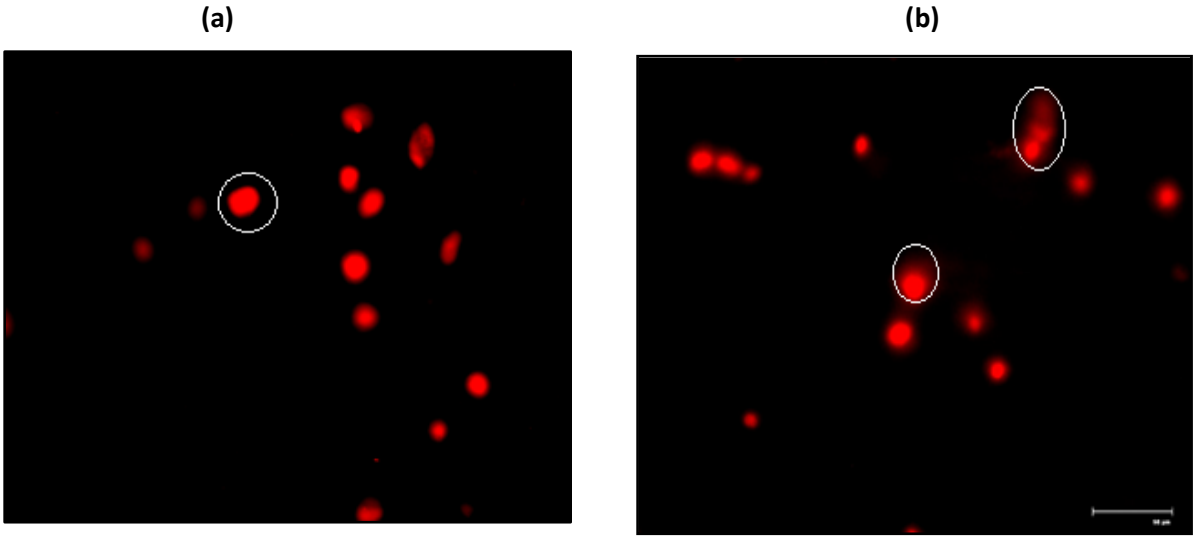

Fig. 15: Single cell gel electrophoresis of HeLa cells (a) Cells in absence of MPTI (b) Cells in presence of MPTI

4.16. Molecular Docking analysis of purified trypsin inhibitor

The computational analysis was also done for the purified Bowman Birk type of protease inhibitor to explore its 3D structure. The Ramachandran plot of MPTI (Fig. 16), and 3-dimensional structure (Fig. 17) of the MPTI has been predicted as shown on Expasy web server. Further, the MPTI was analyzed for protein–protein interaction on Modellar software against trypsin (Fig. 18), which revealed that MPTI has similar inhibitory sites as that of serine protease inhibitor.

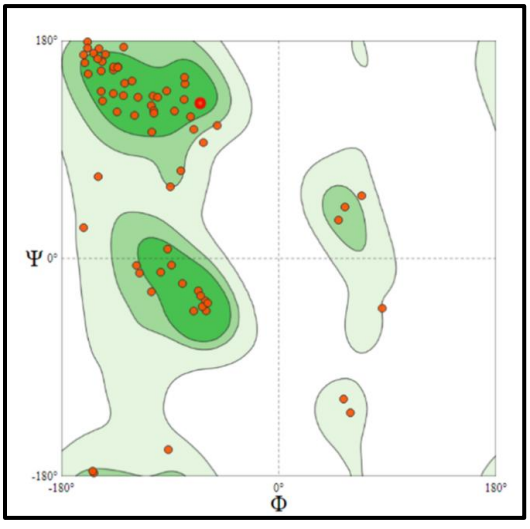

Fig. 16: Predicted Ramachandran plot of the purified MPTI

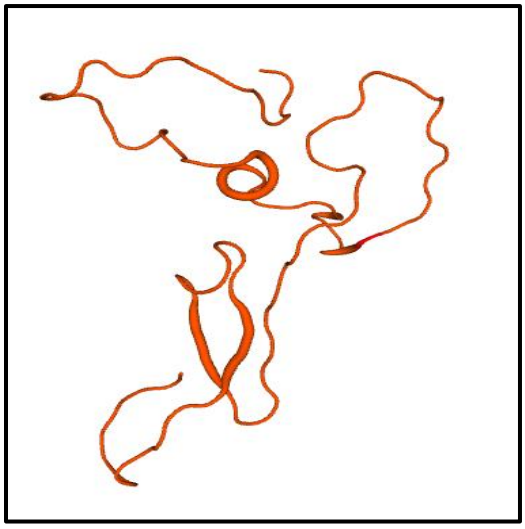

Fig. 17: Predicted 3-D structure of the purified MPTI

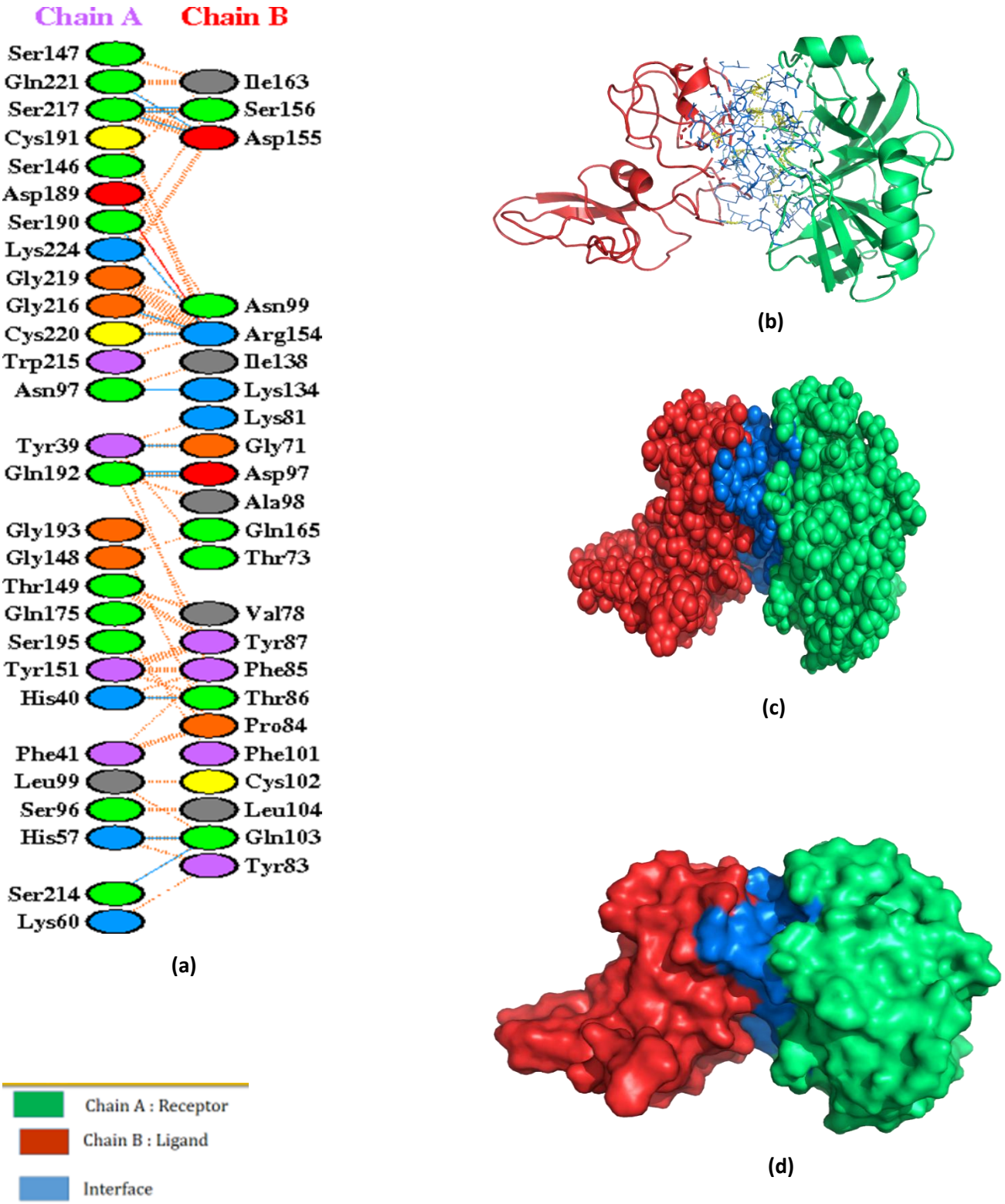

Fig. 18: Molecular Docking analysis of the purified MPTI (a): MPTI-Trypsin interactions; (b, c, and d): Different poses of surface to surface interactions

The current approach available for treating various diseases linked to proteases especially trypsin involves use of chemically processed drugs. The use of these drugs for longer time causes severe side effects. Therefore, an alternative approach of treating such diseases is necessary to use drugs with no side effects, one such approach is to develop natural therapeutic agents. Since, trypsin regulates various process especially pancreatitis and metabolic activities, therefore, it is necessary to regulate the over activity of the trypsin to prevent the pancreatitis which indirectly helps in regulating various metabolic disorders like obesity and diabetes. Further, the purified MPTI from current study can be used to treat the cervical cancer which can helps in the regulating the progression and metastasis of the cancer at primary stage. Hence, it may be presumed that the trypsin inhibitor isolated, purified and characterized from *Mucuna pruriens* in present study may help in regulating the satiety hormones, unwanted proteolytic action in the physiological process, and can help in treating various diseases linked to the metabolism, inflammation and cancer and thus may prove as an ideal candidate for the development of a natural therapeutic drug.

Therefore, the potential activity of this purified trypsin inhibitor from *Mucuna pruriens* could play a vital role in the management of diabetes and nutritional related disorders. It may also be used to treat various inflammations and may prove as a natural therapeutic agent against Cancer. *Mucuna pruriens*, an under-utilized legume found in different regions of the tropical world can also be cultivated as food and might be used as a potential source of natural proteins and further extraction of these beneficiary proteins/protease inhibitors in turn finds its applications in food and pharmaceutical industries.

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Papers published

1. Jafar Khaliq Lone, Lekha MA, Fasil Ali, Rajiv P. Bharadwaj, K S Chandrashekharaiah. Comparison of Total Soluble Protein in Various *Mucuna* species/varieties and Evaluation their role in trypsin inhibition. **Applied Biological Research** 2021, 23(1): 13-20.

Papers presented in conferences

1. Jafar Khaliq Lone, Lekha M A, Rajiv Bhardwaj P, Fasil Ali, K S Chandrashekharaiah. Study of Stability, Kinetic studies and Anti-inflammatory activity of *Mucuna pruriens* trypsin inhibitor. National conference on Recent Novel Approaches in Chemical Science, held at FMKMC College Madikeri, Karnataka on 12th Feb. 2020. **[Poster presentation]**

2. Jafar Khaliq Lone, Lekha M A, Rajiv Bhardwaj P, Fasil Ali, K S Chandrashekharaiah. Identification of multimeric forms of Trypsin inhibitor by using Liquid chromatography coupled to mass spectrometry, purified from the seeds of underutilized legume, *Mucuna pruriens*. National seminar on Emerging trends in Analytical Chemistry (ETOS-2019) held at CSIR-IICT Hyderabad in association with Indian society of Analytical scientists (ISAS) on 21st June, 2019. **[e-Poster presentation]**

3. Jafar Khaliq Lone, M A Lekha, Fasil Ali, Rajiv Bhardwaj, K S Chandrashekharaiah Anti-inflammatory activity of Trypsin Inhibitor isolated and purified from the seeds of underutilized legume "*Mucuna pruriens*". National symposium on Emerging trends and challenges in Life science held by Dept. of Biochemistry, Davangere University on 26th – 27th April, 2019. **[Oral presentation]**

4. Jafar Khaliq Lone, M A Lekha, Fasil Ali, Rajiv Bhardwaj, K S Chandrashekharaiah Isolation and purification of Protease inhibitors from the seeds of underutilized legume "*Mucuna pruriens*". National conference on Synthetic and Biological peptides: Structure and Strategies for the Development of Drugs, Biologics and materials, held Department of studies in Chemistry, Central campus, Bangalore University, Karnataka on 14th - 15th March 2019. [**Poster presentation]**

5. Jafar K. Lone, M A Lekha, Fasil Ali, Sheikh Mansoor, K S Chandrashekharaiah. Antibacterial Activities of Protease Inhibitors of a variety of *Mucuna pruriens* seeds on Some Pathogens. National Inter-disciplinary Conference on Science in development-2018, held in Sri Pratap College, Srinagar on 3rd - 4th Dec. 2018. **[Poster presentation]**

6. Jafar Khaliq Lone, M A Lekha, Fasil Ali, Rajiv Bhardwaj, K S Chandrashekharaiah Identification and Characterization of Trypsin specific Protease inhibitors from the seeds of underutilized legume *Mucuna pruriens*. 39th IABMS international conference on Current Concepts on the role of Indian medicine and phytoceuticals in maintenance of health, held at Dept. of Biochemistry, Jnana Kaveri Campus, Mangalore University from 15-17th Nov. 2018. **[Oral presentation]**

Awards

Best paper presentation award for oral presentation in 2 days national symposium (Emerging trends and challenges in Life science) held by Dept. of Biochemistry, Davangere University on 26th – 27th April, 2019.

Signature of candidate

Signature of Research supervisor